

# Inhibitory Activity of *Artemisia spicigera* Essential Oil Against Fungal Species Isolated From Minced Meat

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## Abstract

**Background:** Meat is an important source of several nutrients. The capability top of fresh meat to rot, causing the group of studies food science, biological and chemical stability meat consideration.

**Objectives:** This study was conducted to examine the inhibitory effect of *Artemisia spicigera* essential oil against fungal species isolated from minced meat.

**Materials and Methods:** Two types of media dichloran 18% glycerol (DG18) agar and dichloran rosebengal chloramphenicol (DRBC) agar were selected for the mycological analysis of the minced meat samples. To evaluate the antifungal activity of essential oils, the microdilution broth method based on the CLSI (M27A) guideline was used.

**Results:** *Artemisia spicigera* essential oil has an inhibitory effect on the growth of fungi found in samples of minced meat. *Aspergillus*, *Penicillium* and *Cladosporium* were the most common genera on both medium types. Average Minimum Inhibitory Concentration<sub>50</sub> = 1.88 µL/mL and MIC<sub>90</sub> = 2 µL/mL were reported. The genus of *Mucor* with MIC = 1.0 µL/mL was the most sensitive and *Aspergillus versicolor* was the most resistant species to the essential oil with MIC = 4 µL/mL.

**Conclusions:** The results of the present study show a favorable inhibitory effect of *Artemisia spicigera* essential oil on fungal growth, especially *Aspergillus* species. According to the results, antifungal components of *Artemisia spicigera* in different forms are used to prevent fungal pollution.

**Keywords:** Antifungal Agent, Food Contamination, Meat, *Artemisia*

## 1. Background

Medicinal plants are nature's gift to human beings to help them pursue a disease-free healthy life, and thus can play an important role in preserving health. Plants have been used as drugs by humans since thousands of years ago. Today, all the world's cultures have an extensive knowledge of herbal medicine. Traditional medicine is based on beliefs and practices that existed before the development of so-called modern medicine or scientific drug therapy. These practices are part of a country's cultural heritage and are transmitted orally or by written transmission. At present, approximately 3,000 Essential Oils (EOs) are known, 300 of which are commercially important, especially for the pharmaceutical, agricultural, food, sanitary, cosmetics and perfume industries. Essential oils or some of their components are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservatives and additives and as natural remedies (1).

Chemically, the EOs consists of terpene compounds (mono-, sesqui- and diterpenes), alcohols, acids, esters,

epoxides, aldehydes, ketones, amines and sulfides. The antimicrobial or other biological activities of EOs are directly correlated to the presence of their bioactive volatile components (2).

Essential oils have been largely employed for their properties which are already observed in nature. Known for their antiseptic (antibacterial, antiviral and antifungal agent) and medicinal properties and their fragrance, they are used in embalmment, preservation of foods, and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies (1).

Due to the climate and vegetation diversity in Iran, identification of effective ingredients of native plants and their extraction for mass production on an industrial scale are of great importance (3).

*Artemisia* L. is a genus of small herbs and shrubs found in northern temperate regions. It belongs to the important family Compositae (*Asteraceae*), one of the most numerous plant groupings, which comprises about 1,000 genera and over 20,000 species. Within this family, *Arte-*

*misia* is included in the tribe Anthemideae and comprises over 500 species, which are mainly found in Asia, Europe and North America. They are mostly perennial herbs dominating the vast steppe communities of Asia. Asia has the greatest concentration of species, with 150 accessions for China, 174 in the ex-Union of Soviet Socialist Republics, about 50 reported for Japan, and 35 species of the genus found in Iran. *Artemisia* species are frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammation and infections by fungi, bacteria and viruses (1).

*Artemisia* species, widespread in nature, are frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammation, and infections by fungi, bacteria and viruses. Many species have been used since ancient times as folk remedies for some treatment purposes (reducing phlegm, relieving cough, invigorating blood circulation, stopping pain, inducing sweat, diuresis, anti hypertension, anti helminthic, antitoxic and antiallergic). The Iranian species has been investigated chemically and the presence of monoterpenes, sesquiterpenes, especially sesquiterpene lactones and essential oils was reported. In fact, the Iranian *Artemisia* spp. has yielded a considerable amount of new, interesting terpenoids (4).

Meat is an important source of several nutrients. It is particularly rich in high biological value protein, as well as micronutrients like iron, selenium, zinc and vitamin B12. Offal meats like liver are also crucial sources of vitamin A and folic acid. Meat is an excellent source of several vitamins and minerals. Red meat provides around 25% of the recommended dietary intakes for riboflavin, niacin, vitamin B6 and pantothenic acid per 100 g and practically two thirds of the daily requirement (DR) of vitamin B12 in the same serving. Meat is also one of the best sources for zinc, selenium phosphorus and iron (5).

Meat spoilage is a complex event, in which a combination of biological and chemical activities may interact and render the product unacceptable for human consumption. Besides lipid oxidation and autolytic enzymatic reactions, spoilage of meat can be considered the result of microbial activity of a wide variety of microorganisms because meat nutrient composition, pH (5.5 to 6.5) and high moisture content allow the growth and survival of a large range of microorganisms (6).

Main spoilage bacteria including *Pseudomonas*, *Moraxella*, *Lactobacillus*, *Leuconostoc*, *Proteus* species and etc, yeasts and molds can be grown in meat (7). Molds are ubiquitous biological agents that are able to colonize foods because of their potential to synthesize a wide diversity of hydrolytic enzymes (8).

The Capability top of fresh meat to rot, causing the group of studies food science, biological and chemical stability meat consideration (9).

## 2. Objectives

Until now many studies have been conducted in the field of antibacterial essential oils *Artemisia* species. How-

ever, no study has been done on the effect of antifungal *Artemisia* species, Sagebrush, and *Artemisia spicigera*.

## 3. Materials and Methods

### 3.1. Plant Material and Essential Oil Isolation

The flowering aerial parts of *Artemisia spicigera* were collected from Bostanabad area of East Azerbaijan province. The preparation of the extract was conducted at the department of botany, faculty of pharmacy, Tabriz University of Medical Sciences. The parts collected from the fresh aerial plant material were subjected to hydro-distillation using a Clevenger type apparatus for 3 hs according to the procedure described in the European Pharmacopoeia. Anhydrous sodium sulphate was used to remove water after extraction. The essential oil was stored in an air tight glass container at 4°C.

### 3.2. Sample Collection

A random 80 minced meat samples (20 samples per season from distribution centers) were collected aseptically from different markets in sterile bags. After the collection, the samples were transported to mycology laboratory, University of Tabriz for immediate analysis. The samples were analyzed for total fungal count and identification of fungal species.

### 3.3. Isolation of Fungi

Two types of media were chosen for the mycological analysis of the meat samples (10). The medium dichloran rosebengal chloramphenicol (DRBC) agar contains (g/L): peptone 5 g, glucose 10 g,  $\text{KH}_2\text{PO}_4$  1 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, dichloran solution (0.2% (w/v) in ethanol) 1 mL, Rose Bengal 0.025 g, chloramphenicol 0.1 g, agar 15 g, and distilled water 1000 mL. Final pH should be 5.6. Ingredients are mixed, heated to dissolve agar and sterilized by autoclaving at 121°C for 15 minutes. The medium is allowed to cool to 50°C in a water bath prior to pour plating. The Dichloran Rosebengal chloramphenicol agar is especially useful for analyzing sample containing spreader molds (e.g. *Mucor*, *Rhizopus*, etc.), since the added Dichloran and Rosebengal effectively slow down the growth of fast-growing fungi, thus readily allow detection of other yeast and mold propagules, which have lower growth rates. The medium Dichloran 18% Glycerol agar comprises (g/L): peptone 5 g, glucose 10 g,  $\text{KH}_2\text{PO}_4$  1 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, dichloran (0.2% in ethanol) 1 mL, glycerol 220 g, chloramphenicol 0.1 g, agar 15 g, and distilled water 1000 mL. These media were autoclaved at 121°C for 20 minutes. The final pH should be 5.6 and the final aw, 0.955. This medium is used as a general purpose mold enumeration medium.

Then, 1 g of each sample was aseptically transferred to homogenizer flasks containing 9 mL of sterile peptone water (0.1%) and homogenized at 1,400 r.p.m. for 2 min-

utes. To provide  $10^{-1}$  dilution, serial dilutions were made up to  $10^{-20}$  and  $10^{-100}$ . Each dilution was spread over two plates of the Sabouraud's dextrose agar supplemented with chloramphenicol and first inspected after 7 days incubation at  $25^{\circ}\text{C}$  for fungal growth degree with final report on the 7th day. All moulds were identified according to standard procedures as macroscopic and microscopic examination using slide cultures. Whereas all yeast isolates were identified by the relevant methods as morphological and microscopical studies, on differential culture media, were cultured and isolated.

### 3.4. Diagnosis of Fungal Genus and Species

Colonies grown in the medium were identified to species based on their morphology and microscopic characteristics were used to identify the different fungal isolates contaminating the meat samples. To ensure the strains of fungi, morphologic diagnosis was made based on macroscopic and microscopic approaches.

### 3.5. Macroscopic and Microscopic Methods

Selected fungal colonies were identified to species level based on macroscopic morphology and microscopic features (11).

Colony characters and diameters on specific media are important features for species identification. Morphology is the physical architecture through which an organism functions in and adapts to its environment; however, some aspects may vary or be induced by specific cues in the immediate environment. As a result, strains characterized in one laboratory might look different when grown in another because of subtle differences in nutrients, temperature, lighting or humidity. This sometimes makes comparisons between different studies very difficult. These effects can be minimized using strictly standardized working techniques for medium preparation, inoculation technique and incubation conditions (12).

Morphology forms an important part of the fungi species concept. Colony characters used for characterizing species include colony growth rates, texture, degree of sporulation, production of sclerotia or cleistothecia, colors of mycelia, sporulation, soluble pigments, exudates, colony reverses, sclerotia, Hülle-cells and cleistothecia (13).

For this purpose, fungal isolates were cultured on different media. All isolated fungi at the media Czapek's (CZ) agar, Yeast Extract Sucrose (YES) agar and Sabouraud's dextrose agar were cultured, and incubated at the standard temperature of  $25^{\circ}\text{C}$  for 7 days. It is crucial that temperatures are carefully checked as small differences have a large impact on colony growth (12).

Depending on the circumstances and the type of fungal isolates obtained from other functional areas such as environment, the creatine-sucrose agar, malt extract agar, corn meal agar and chromium agar were used in the detection of fungi (14).

Microscopic diagnosis by direct smear microscopy

(tease mount) of fungal colonies grown in the Said medium or by using the technique slide culture was performed according to standard procedures described. All microscopic slides with lactophenol blue color were prepared and preserved using a special adhesive silk. Microscopic characteristics, using manual keys detect fungi, for definitive diagnosis, were coordinated.

### 3.6. Determination of Antifungal Activity of Essential Oils

To evaluate the antifungal activity of essential oils, a microdilution method was used. This method was performed in microplates 96 in volume  $200\ \mu\text{L}$ . Double concentration of Roswell Park Memorial Institute 1640 medium with glucose and 3-(N-morpholino) propanesulfonic acid in 900 mL tissue culture grade water with constant, gentle stirring until the powder is completely dissolved. After it has dissolved completely, the pH can be raised to 7 with 10 M Sodium hydroxide, sterilize the medium immediately by filtering through a sterile membrane filter with a porosity of  $0.22\ \mu\text{m}$ . Store liquid medium at  $4^{\circ}\text{C}$  and in dark till use. The 2 xRPMI 1640 solution at  $4^{\circ}\text{C}$  is stable for several weeks.

The essential oils were serially diluted ( $0.5 - 10\ \mu\text{L/mL}$ ). A working dilution of essential oil 1x RPMI was prepared that the concentration of this stock should be double the highest concentration in the range of measurement. After shaking, 200 mL of the pharmaceutical solution was added to the first column wells of 96-micro titer plates, and in columns 2 and 12, 100 mL of the 1 x RPMI medium was added.

To prepare the inoculums, some of fungal colonies in 5 mL of water were solved. Suspension must be thoroughly mixed and vortex. Then number of fungal cells in suspension using a hemocytometer (Neubauer) counts, so that by taking 10.5 mL the appropriate number from fungal cell per well can be achieved.

Plates were incubated in  $30^{\circ}\text{C}$  for 48 - 24 hours. Minimum Inhibitory Concentration can be examined visually, or can be read using a spectrophotometer. (wavelength 540 nm). To read with a spectrophotometer from column 12 as Blank should be used.

The lowest concentrations without visible growth were defined as the minimal concentrations, which completely inhibited fungal growth (MIC). Ninety percent and 50% inhibition rates of colony growth were MIC90 and MIC50, respectively.

## 4. Results

Mycological qualification of such mince has very important usage. This study reported 100% of meat samples, collected from different retail markets were contaminated with molds. In fact, of the 80 samples was at least a genus or species of the fungal culture was obtained. Pollution severity in the number of fungal colonies and the number of species has been isolated in autumn than

in other seasons (50 isolates of the fungus) and in winter this amount is lower (23 isolates of the fungus).

The mycological analysis of the 80 samples of mince revealed the isolation of 10 fungal species (Tables 1 and 2). The total fungal population on DG18 was relatively higher than that on DRBC (145 versus 132 colonies/ g mince, al-

though fungal colony was greater in DRBC). *Aspergillus*, *Penicillium* and *Cladosporium* were the most common genera on both medium types.

The frequency of total fungi isolated from Media DG18 and DRBC was shown in Figure 1 in different seasons. Figure 1 shows a higher rate of infection in autumn.

**Table 1.** Frequency of Fungal Genera and Species Isolated From Mince Samples on Dichloran Rosebengal Chloramphenicol Agar (DG18) in Different Seasons<sup>a</sup>

Candida Species	Spring	Summer	Fall	Winter	Total
<i>Aspergillus flavus</i>	60 (12)	50 (91)	65 (13)	35 (7)	52.5 (42)
<i>Aspergillus niger</i>	10 (2)	20 (4)	30 (6)	10 (2)	17.5 (14)
<i>Aspergillus terreus</i>	5 (1)	0 (0)	0 (0)	0 (0)	1.25 (1)
<i>Aspergillus versicolor</i>	0 (0)	0 (0)	5 (1)	0 (0)	1.25 (1)
Other <i>Aspergillus</i> Species	10 (2)	10 (2)	15 (3)	0 (0)	8.75 (7)
Genus <i>Fusarium</i>	0 (0)	5 (1)	5 (1)	0 (0)	2.5 (2)
Genus <i>Mucor</i>	0 (0)	5 (1)	10 (2)	5 (1)	5.25 (5)
Genus <i>Rhizopus</i>	5 (1)	10 (2)	15 (3)	0 (0)	7.5 (6)
Genus <i>Rhizomucor</i>	0 (0)	0 (0)	5 (1)	0 (0)	1.25 (1)
Genus <i>Cladosporium</i>	20 (4)	25 (5)	30 (6)	20 (4)	23.75 (19)
Genus <i>Penicillium</i>	55 (11)	45 (9)	50 (10)	30 (6)	45 (36)
<i>Trichothecium</i>	0 (0)	5 (1)	0 (0)	0 (0)	1.25 (1)
<i>Alternaria</i>	0 (0)	5 (1)	5 (1)	10 (2)	6.25 (5)
Yeast	10 (2)	10 (2)	15 (3)	0 (0)	8.75 (7)
<b>Total</b>	<b>24.1 (35)</b>	<b>26.2 (38)</b>	<b>34.5 (50)</b>	<b>15.2 (22)</b>	<b>100 (145)</b>

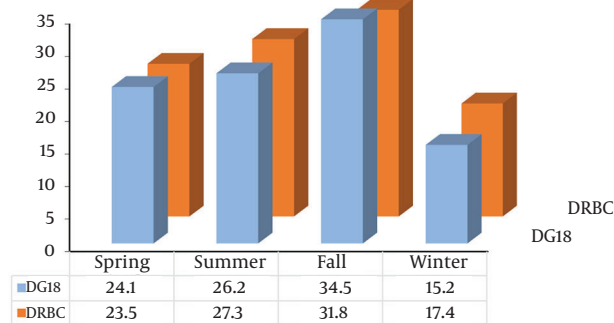
<sup>a</sup>Values are presented as % (No.).

**Table 2.** Counts of Fungal Genera and Species Isolated From Mince Samples on Dichloran Rosebengal Chloramphenicol Agar in Different Seasons<sup>a</sup>

Candida Species	Spring	Summer	Fall	Winter	Total
<i>Aspergillus flavus</i>	50 (10)	45 (9)	50 (10)	30 (6)	43.75 (35)
<i>Aspergillus niger</i>	10 (2)	15 (3)	30 (6)	5 (1)	15 (12)
<i>Aspergillus versicolor</i>	0 (0)	0 (0)	5 (1)	0 (0)	1.25 (1)
Other <i>Aspergillus</i> Species	10 (2)	5 (1)	10 (2)	0 (0)	6.25 (5)
Genus <i>Fusarium</i>	0 (0)	10 (2)	10 (2)	5 (1)	6.25 (5)
Genus <i>Rhizopus</i>	5 (1)	5 (1)	5 (1)	0 (0)	3.75 (3)
Genus <i>Cladosporium</i>	25 (5)	30 (6)	30 (6)	25 (5)	27.5 (22)
Genus <i>Penicillium</i>	50 (10)	60 (12)	50 (11)	30 (7)	50 (40)
<i>Ulocladium</i>	0 (0)	0 (0)	0 (0)	5 (1)	1.25 (1)
<i>Alternaria</i>	0 (0)	10 (2)	10 (2)	10 (2)	7.5 (6)
Yeast	5 (1)	0 (0)	5 (1)	0 (0)	2.5 (2)
<b>Total</b>	<b>23.5 (31)</b>	<b>27.3 (36)</b>	<b>31.8 (42)</b>	<b>17.4 (23)</b>	<b>100 (132)</b>

<sup>a</sup>Values are presented as % (No.).





**Figure 1.** The Frequency of Total Fungi Isolated From Media DG18 and DRBC at Each Season

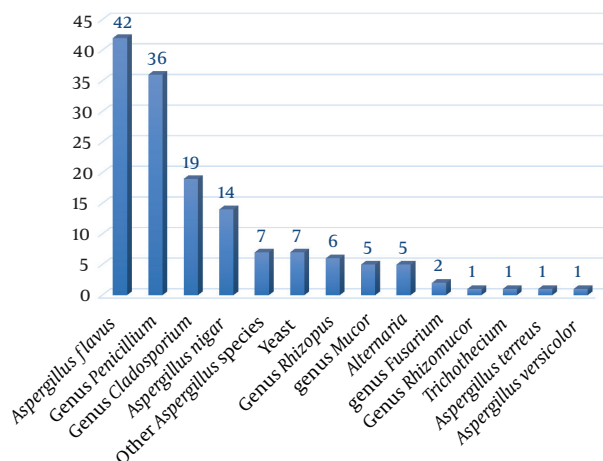
On the DRBC medium, *Penicillium*, 50% of total population, *Aspergillus* species, 66.25% of total population and *Cladosporium*, 27.5% of total population was the major fungus contaminating all samples recording the highest total count among all fungi. Among the four species of *Aspergillus*, *Aspergillus flavus* was the commonest (52.5% of samples) followed by *Aspergillus niger* (17.5% of samples) and *Aspergillus terreus* (1.25% of samples) and other species of *Aspergillus* (8.75% of samples).

The remaining fungal genera and species were less frequently encountered from mince and were represented by *Fusarium*, *Rhizopus* genus, *Ulocladium*, *Alternaria* and Yeasts.

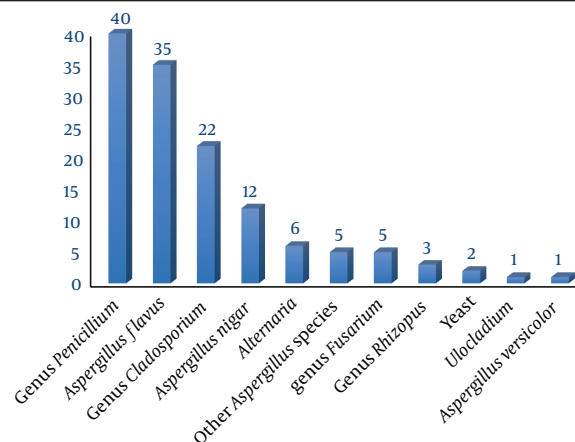
Utilization of G18 medium revealed the isolation of 14 fungal species belonging to four genera of which *Rhizomucor*, *Mucor*, *Trichothecium*, *Aspergillus terreus* and unidentified yeast species were only encountered on this medium and were not isolated on DRBC. Figures 2 and 3 have shown the total frequency of fungi isolated from the DG18 and DRBC media.

Presence of pathogenic fungi and mycotoxins is significant. Isolation of *Aspergillus flavus* and *Aspergillus versicolor* is also significant. The DRBC medium inhibits the growth of mucorales genera; therefore, different fungal genus or species might be found. However, in the present study, we obtained more genera and species in DG18 medium.

Minimum Inhibitory Concentration of the essential oils was variable depending to species of fungi. The genus of *Mucor* with MIC = 1.0  $\mu\text{L/mL}$  was the most sensitive and *Aspergillus versicolor* was the most resistant species to the essential oil with MIC = 4  $\mu\text{L/mL}$ . Since growth inhibition of studied essential oils was evident in this study, they have potential to control of these article fungi and could be considered for developing new antifungal agent (Table 3).



**Figure 2.** Total Frequency of Fungi Isolated From Dichloran 18% Glycerol Medium



**Figure 3.** Total Frequency of Each Fungi Isolated From the Dichloran Rose-bengal Chloramphenicol Medium

**Table 3.** Inhibitory Effects of Essential Oil of *Artemisia* (*Artemisia spiciger*) on Fungal Species Isolated From Mince

Candida Species	MIC50 ( $\mu\text{L/mL}$ )	MIC90 ( $\mu\text{L/mL}$ )
<i>Aspergillus flavus</i>	2.5	2.5
<i>Aspergillus niger</i>	2.0	2.5
<i>Aspergillus terreus</i>	2.5	2.5
<i>Aspergillus versicolor</i>	4	4
<i>Genus Fusarium</i>	2.5	2.5
<i>Genus Mucor</i>	1.0	1.25
<i>Genus Rhizopus</i>	1.25	1.25
<i>Genus Rhizomucor</i>	1.25	1.25
<i>Genus Cladosporium</i>	2.5	2.5
<i>Genus Penicillium</i>	1.25	2
<i>Trichothecium</i>	1.25	1.25
<i>Alternaria</i>	1.25	1.25
Yeast	1.25	1.25
Average	1.88	2

Abbreviation: MIC, minimum inhibitory concentration.

## 5. Discussion

This study showed that the fungal load per sample might be correlated with the type of medium on which the samples were cultured. The two isolation media (DRBC and DG18) showed to be suitable for culturing food borne fungi contaminating meat samples. The sources of such contaminants are uncertain, as it is difficult to predict whether the contamination happened at the origin of production or during processing. According to increase in fungal species in autumn toward other seasons, this could be due to the particular weather conditions in this season, there is more dust, reduce the heat and to some extent proper temperature and humidity for the growth of fungi.

Until now, there were no reports on the effects of essential oils of *Artemisia spicigera* on fungi species in minced meat. Based on our findings, *Artemisia spicigera* essential oil affected the growth inhibition of the studied fungi. In all studied fungi, the essential oils caused growth inhibition. In other words, the effects of growth inhibitory of plant essential oils depend on species of fungi (11).

It could be seen that as essential oil concentrations were increased, the inhibitory effect was also increased. In other words, the inhibitory effect of the essential oils was proportional to its concentration. However, with increased concentrations of essential oil, the susceptibility of fungi was increased (11).

Theoretically, climate change could impact on meat safety as well as organoleptic quality, especially if the animals carry more enteric pathogens in their gut or on their body surface (15). Climatic conditions will determine the establishment and growth of a microorganism. The microflora of food comprises microorganisms associated with raw materials, those acquired during handling and processing and those surviving preservation and storage (16).

The contamination of meat by bacteria and fungi pose threat to its consumption by humans. Consumption of contaminated meat may lead to food poisoning (17).

The presence of fungal units (spores, hyphae or budding cells) can be expected as contamination from the environment during meat processing. The presence of fungal elements in samples reveals the presence of unsanitary condition in the meat distribution centers and sales of meat and their numbers were considered to be a more practical indicator of the hygienic efficiency and microbiological status of mince.

These results of the present study confirmed the reports of previous researchers who studied about this subject. This shows the difference of inhibitory potential of these components on different fungi. The results show a favorable inhibitory effect of *Artemisia spicigera* essential oil on fungi growth. According to this result, antifungal components of *Artemisia spicigera* in different forms can be used to prevent fungal pollution (18).

In a study, 100 samples of imported frozen meat collect-

ed from different Alexandria markets were investigated both quantitatively and qualitatively for fungal burden. This study reported that 92% of meat samples collected from different retail markets in Alexandria were contaminated with molds. High count of mold and yeast in this study has been reported due to poor sanitary measures during production, transportation, handling and storage of imported frozen meat (19).

In Katirae et al. study, antifungal activity of *Artemisia* against isolates of *Candida albicans* was drug-resistant. In this study, the mean concentration of growth inhibition was 1.18 µL/mL (20).

According to the obtained results, essential oils of *Artemisia spicigera* represent a good basis for the formulation of products with potential efficacy in the control of fungi. The results indicate that all tested fungi were relatively uniformly susceptible.

The results indicate that fungal elements were found in mince in distribution centers and sales of meat. This may pose a real danger to consumers, since mince are readily accessible and very convenient. There is clearly a need to improve quality.

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## Footnotes

**Authors' Contribution:** Peyman Ghajarbeygi, Narges Saki, Farzad Katirae and Razzagh Mahmoudi developed the original idea and protocol, abstracted and analyzed data, and wrote the manuscript.

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